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14. ABSTRACT
A significant number of lung cancer amplicons were established between mid and late 2000s using the array-based genomic tools. Thus, the known gene compositions of many MRAs are devoid of the genes only recently discovered. Exemplary groups of such genes include many non-protein-coding genes like long non-coding RNAs and microRNAs (miRNAs). In study, we examined the PVT1-encoded miRNAs embedded in one of the most recurrent amplicons at 8q24. The results indicate that these miRNAs are not likely functionally relevant to lung tumorigenesis. The implication is that future studies of the 8q24 amplicon should concentrate on characterizing the PVT1 RNA transcript.

15. SUBJECT TERMS
Noncoding RNA, microRNA, lung cancer, Genome annotation

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1. Introduction

Gene amplification is a common form of genetic alterations in lung cancers. Many *bona fide* lung cancer genes undergo gene amplification for activation. An event of gene amplification often covers a long segment of human genome, encompassing many genes. This makes it difficult to assign functionally important target genes, i.e. driver genes, within a multigenic amplicon. A strategy to alleviate this issue is to identify the **m**inimal **r**egion of **a**mplification (**MRA**) that is consistently amplified across the tumors examined. Every gene within an MRA is a candidate oncogene and the expectation is that establishing an MRA would minimize the number of candidate oncogenes to be validated by follow-up studies. While this is true, cancer researchers often ignore this reality that complicates our understanding of the known gene content of an MRA, i.e. **the known gene content of an MRA may increase with time due to the fact that new functional elements continue to be discovered in the human genome (1)**. A significant number of lung cancer MRAs were established between mid and late 2000s using the array-based genomic tools (2). Thus, the known gene compositions of many MRAs are devoid of the genes only recently discovered. Exemplary groups of such genes include many non-protein-coding genes like long non-coding RNAs (lncRNAs) and microRNAs (miRNAs). Intriguingly, certain noncoding RNAs and other recently discovered genes reside in known MRAs of recurrent lung cancer amplicons. We term them "**G**enes **N**ew **T**o **E**xisting **M**RAs (**GNTEMs**)". Our hypothesis is that GNTEMs harbor functionally critical players in lung cancer initiation and progression. In this study, we will investigate GNTEMs embedded in one of the most recurrent lung cancer amplicons for functional roles in lung cancers.

2. Keywords

Noncoding RNA, microRNA, Lung cancer, Oncogene.

3. Accomplishments

There are two specific aims to test the hypothesis of the proposed study:
1. Functional analysis of GNTEMs for driver oncogene properties in cell-based systems.
2. Functional analysis of GNTEMs for driver oncogene properties in animal-based systems.
Below, we break down the progresses of each Specific Aim.

3.1 Major goals of the project

3.1.1. Major Task 1: Functional analysis of GNTEMs for driver oncogene properties in cell-based systems.
There are three subtasks associated with this Major Task. The progresses produced under each Subtask are classified below:

3.2 What was accomplished under the major goals?

3.2.1 Subtask 1: Acquisition of human lung cancer cell lines from ATCC and RNA expression analysis of the GNTEMs (miR-1204~1208) in a panel of human lung cancer cell lines cells.
We acquired these human lung cancer cell lines to facilitate this project: NCI-H1792, NCI-H23, NCI-H441, and NCI-H3122. Both NCI-H1792 and NCI-H23 cell lines harbor the 8q24 MYC amplicon, whereas NCI-H441 and NCI-H3122 do not. MicroRNAs (miRNAs) of these cell lines were isolated using the mirVana miRNA isolation kit (Thermo Fisher Scientific) and the RNA expression levels of miR-1204 ~ 1208 were quantified using the Taqman miRNA assays developed at Thermo Fisher Scientific, with the RNU44 expression as the normalization reference across all samples. We also included the premalignant human lung epithelial cells (BEAS-2B) as a reference point. As shown in **Fig 1**, three of the five miRNAs were found expressed in the human lung cancer cells. In particular, the 8q24-amplified NCI-H1792 cells appeared to harbor a higher expression of miR-1206 in comparison with the BEAS-2B reference cells and the two cell lines lacking the 8q24 amplification (NCI-H441 and NCI-H3122). Intriguingly, none of the cells analyzed appeared to express

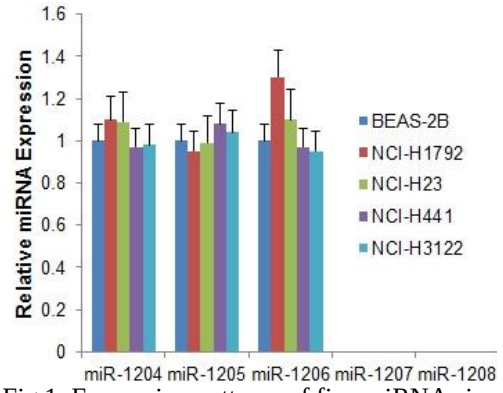

Fig 1. Expression patterns of five miRNAs in human lung cancer cells.

miR-1204: UCCAUUAUUUGAGAUGAGUUAC
miR-1205: UGCAGGGUUUGCUUUGAGGUAC
miR-1206: GAUGUUUAAGCUCUUGCAGUAG
miR-1206: AGUGUUCAUGUAGAUGUUUAAG
miR-1206: CAGUGUUCAUGUAGAUGUUUAA

Fig 2. siRNAs designed to target individual miRNAs. The corresponding shRNAs were designated: sh1204, sh1205, sh1206a, sh1206b, and sh1206c.

miR-1207 and miR-1208. In a published study by Beck-Engeser et al. (4) to examine the five predicted miRNAs encoded by *PVT1* at 8q24 in T cells, miR-1208 was the weakest in terms of supporting evidence as a bona fide miRNA. Our results are in line with the notion that miR-1207 and miR-1208 may be associated with a very narrow expression spectrum in human tissues (outside the lung tissue).

3.2.2 Subtask 2: Establishing stable suppression of individual GNTEMs in two amplified human lung cancer cell hosts.

Although the expression patterns of the 8q24 miRNAs in the analyzed lung cancer cell lines were not suggestive of robust alterations, we proceeded with the second Subtask of creating stable suppression of individual miRNAs in the two amplified lung cancer cell lines (NCI-H1792 and NCI-H23). Since miR-1207 and miR-1208 were not expressed in our cell systems, they were not included in the subsequent study. We used the RNAi Central Design Tool (http://cancan.cshl.edu/RNAi_central/RNAi.cgi?type=shRNA) to design siRNAs targeting individual miR-1204, miR-1205, and miR-1206. As shown in **Fig 2**, the RNAi Central Design Tool only identified a single siRNA species each to target miR-1204 and miR-1205. However, for miR-1206, three different siRNAs were identified. Based on these siRNA sequences, we created retroviral expression plasmids, containing the miR-30 backbone, to express these siRNAs. The expressed shRNAs were packaged in the miR-30 backbone which is conducive to efficient processing by the miRNA biogenesis machinery. In addition, we improved the expression vector by modifying them into the "miR-E" form which has been shown by Fellmann et al. to produce a higher level of shRNA expression ((3), **Fig 3**). Stable transfectant cells of NCI-H1792 harboring the individual shRNA expression vectors were obtained following selection using the appropriate antibiotics. By RT-QPCR, we found that all the designed shRNAs were capable of suppressing the intended target miRNAs to different degree (**Fig 4**). In particular, sh1204 and sh1205 knocked down the intended target miRNAs by approximately 60% and 63%, respectively. Two of the three sh1206s conferred > 80% suppression of the target miR-1206. Importantly, the shRNAs did not appear to exhibit off-target effects toward the other two unintended miRNAs.

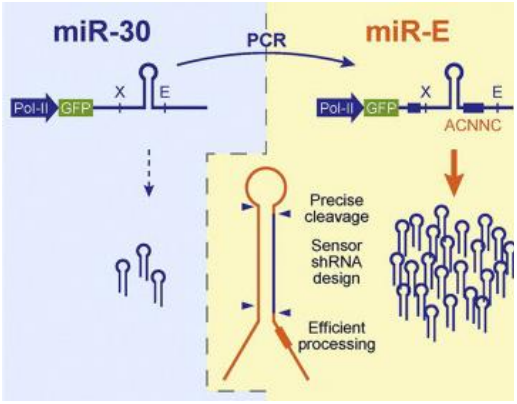

Fig 3. MiR-30-based shRNA expression vector and the improved version, miR-E (3).

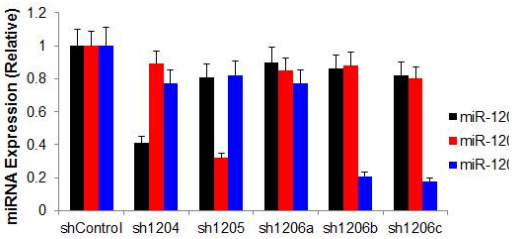

Fig 4. Target miRNA suppression efficiency by the designed shRNAs.

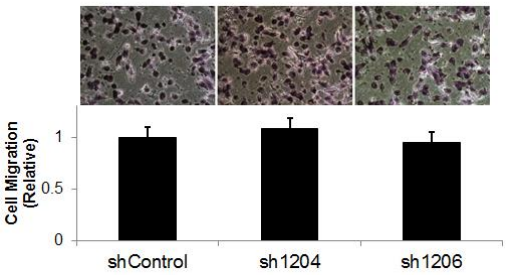

Fig 5. Similar cell migratory potential among the miRNA knockdown cells (transwell migration assay).

3.2.3 Subtask 3: Evaluation of five in vitro cellular phenotypes of the stable knockdown cells.

Five in vitro cellular phenotypes of the miRNA knockdown cells were analyzed. These phenotypes include: a) anchorage-independent growth (soft agar assay), (b) transwell migration assay, (c) transcellular invasion assay, (d) colony formation at low plating density, and (e) apoptosis induced by lack of substrate attachment (anoikis). Surprisingly, none of the miRNA knockdown cells exhibited phenotypic differences from the control cells in these cell-based assays (**Fig 5** and data not shown). Consequently, we conclude that these miRNAs are not likely to mediate the functional consequences of the 8q24 amplicon.

3.2.4 Major Task 2: Functional analysis of GNTEMs for driver oncogene properties in animal-based systems.

The negative cell-based findings under Aim 1 made the research under Aim 2 a moot point. We thus did not initiate the study under Aim 2.

3.3 Opportunities for training and professional development of the project
Nothing to report

3.5 How were the results disseminated to communities of interest?
Nothing to report

3.6 What do you plan to do during the next reporting period to accomplish the goals?
Not applicable. This is the final report.

4. Impact
4.1 What was the impact on the development of the principal discipline(s) of the project?
The 8q24 *MYC* amplicon is one of the most frequently amplified region in all cancer genomes. For a long time, the *MYC* oncogene had always been the presumptive target gene of this gene amplification event. However, data in the literature has repeatedly challenged this notion because the minimal amplicon at 8q24 appears to contain *MYC* and *PVT1*, raising the possibly that *PVT1* may also functionally contribute to the phenotypes associated with the 8q24 amplicon. Indeed, reports have documented evidence that *PVT1* is functionally relevant to the 8q24 amplicon (5,6). However, since the introns of *PVT1* encode miRNAs, an important but unresolved issue is the functional significance of the miRNAs encoded by *PVT1* introns in the cancer biology of the 8q24 amplicon. We chose to investigate this issue in lung cancers in view of the significant knowledge gap regarding *PVT1*-encoded miRNAs in lung cancers. Our results suggest that these miRNA are unlikely to play significant functional roles in the lung cancers carrying the 8q24 amplicon. Future studies will focus on dissecting the mechanistic intricacy of *PVT1* in lung tumorigenesis.

4.2 What was the impact on other disciplines?
Nothing to report.

4.3 What was the impact on technology transfer?
Nothing to report.

4.4 What was the impact on society beyond science and technology?
Nothing to report.

5. Changes/Problems
In this study, the outcome of Aim 1 suggests that the miRNAs of interest are not functionally critical in lung cancer cells, obviating the rationale to pursue the animal studies under Aim 2. Nevertheless, an implication of the study outcome is that future studies to investigate the lung cancer biology of the 8q24 amplicon should focus on the *PVT1* RNA transcripts rather than the miRNAs encoded by *PVT1*.

6. Products
Nothing to report

7. Participants & other collaborating organizations
7.1 Individual who worked on the project

Name	David Mu
Project role	PI
Researcher ID	0000-0002-7762-0182 (ORCID)
Nearest person month worked	2.4
Contribution to project	Project design and cell based assays
Funding support	This award

Name	Shao-Chiang Lai
Project role	Postdoc
Researcher ID	None
Nearest person month worked	2.4
Contribution to project	Functional analyses of miRNA KD cells
Funding support	This award

7.2 Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report

7.3 What other organizations were involved as partners?
Nothing to report

8. Special reporting requirements
Not applicable.

9. Appendices
None.

10. Literature cited

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